치의학박사 학위논문

Effect of S100A4 induction in periodontal ligament cells on osteoclast formation

치주인대세포의 S100A4 발현 증가가 파골세포 형성에 미치는 영향

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마수정

Abstract

Effect of S100A4 induction in periodontal ligament cells on osteoclast formation

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(*Directed by Professor* **Won Hee Lim,** DDS, MSD, PhD)

Objective:

An increase in the expression of S100A4 has been reported in various inflammatory diseases. However, little is known about the association between periodontal inflammation and S100A4 expression. The aims of this study were to investigate changes in S100A4 expression in human periodontal ligament (hPDL) cells in response to inflammatory stimuli and to describe a possible mechanism underlying the change.

Materials and Methods:

Human PDL cells were treated with lipopolysaccharide (LPS) and the level of S100A4 was analyzed by real-time RT-PCR and Western blotting. LPS was added to co-cultures of hPDL and osteoclast progenitor cells under osteoclastogenic

condition and the formation of osteoclasts was assessed. Alternatively, progenitor cells were directly treated with recombinant S100A4 for evaluation of osteoclastogenesis. The activity of nuclear factor kappaB (NFκB) was examined by Western blotting for phosphorylated forms of inhibitorkappaB (IκB) and p65. An NFκB inhibitor was added to the culture of hPDL cells with LPS and the level of S100A4 was measured by real-time RT-PCR.

Results:

LPS stimulation resulted in a significant increase of S100A4 expression in hPDL cells. S100A4 protein secretion from hPDL cells was also increased. The enhanced expression of S100A4 in hPDL cells under inflammatory conditions led to stimulation of the generation of osteoclasts. In addition, direct S100A4 treatment stimulated osteoclastogenesis. The underlying mechanism for the increased S100A4 expression in hPDL cells was activation of the NFκB signaling pathway.

Conclusions:

The results suggest that bone destruction in periodontitis might be associated with increased S100A4 expression in hPDL cells.

Key words: S100A4; periodontal ligament cells; osteoclastogenesis; inflammation; NFκB
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I. INTRODUCTION

S100A4, a 101 amino-acid protein, is a member of the S100 calcium-binding protein family.[1] Expression of S100A4 has been reported in smooth muscle, bone marrow, osteoblast, and normal PDL cells.[2] Expression of S100A4 in PDL tissue is higher than that in other oral tissues such as dental follicle, dental papilla, and gingiva.[3] Interestingly, the PDL cells in erupted teeth exhibit high levels of S100A4 expression, whereas its expression in under-erupted teeth is only faintly detectable.[3]

The expression of S100A4 markedly increases in patients with rheumatoid arthritis (RA), a chronic inflammatory disease that leads to progressive destruction of joint architecture.[4] several studies have reported a marked association between RA and periodontitis.[5-7] Moreover, RA patients have not only an elevated prevalence of periodontitis, but also may have severe advanced periodontitis with aggressive alveolar bone loss.[7-10] Advanced periodontitis may also be observed in RA patients without impaired oral hygiene,[8, 10] and severe periodontal breakdown can occur without any change in oral bacteria flora, as reported in an animal arthritis model study.[11]

Although S100A4 is assumed to affect the function of PDL cells, its precise role in periodontitis remains undescribed. In particular, little has been reported about the relationship between alveolar bone lysis induced by periodontitis and S100A4 expression. However, an association between S100A4 and bone destruction has been suggested in RA. In an arthritis model induced by staphylococcal infection, S100A4 knock-out mice were shown to be protected from subarticular bone destruction.[12] However, the direct regulation of osteoclasts by S100A4 has not yet been demonstrated. Therefore, whether the stimulation of bone erosion by S100A4 in inflammatory conditions is a result of its direct effect on osteoclasts or a consequence of its indirect influence on osteoclasts via regulation of inflammatory factors is not clear. As many aspects are common to periodontitis and RA, it can be hypothesized that S100A4 is involved in periodontal inflammation and osteoclastic bone destruction.

The aims of this study were to investigate changes in S100A4 expression in human periodontal ligament (hPDL) cells during periodontal inflammation, to evaluate the effect of S100A4 on the generation of bone-resorbing osteoclasts, and to explore a possible mechanism involved in the regulation of S100A4 expression.

II. REVIEW OF LITERATURES

S100A4, a low molecular weight (12KDa) acidic protein, is a member of the S100 family of calcium-binding proteins.[1, 13] S100 family members have two EF-hand type calcium-binding motifs; one canonical and another specific to the S100 family (Figure 1).[14] The S100 specific calcium binding motif are involved in several cellular processes such as cell metabolism, motility, and intra-cellular signaling.[15, 16] S100A4 is known to be involved in motility and metastasis of cancer cells by interacting with cytoskeletal components.[17, 18] Therefore, to date, it is considered a prognostic marker for cancer progression and metastasis.[19] It is also reported that S100A4 is expressed in normal tissues such as smooth muscle, liver, bone marrow, smooth muscle cell of arteries, kidney, and osteoblastic cell in humans.[2, 20] S100A4 is expressed in the cells with a mobile phenotype, such as endothelial cells, fibroblasts and leukocytes.[1] However, physiological functions of S100A4 in normal tissues have not been clarified yet.

Most of S100A4 proteins are synthesized and localized intracellularly and exert regulatory role within cells by interacting with their target proteins after binding Ca. S100A4 directly interacts with cytoskeletal proteins such as actin, tropomyosin, and non-muscle myosin, and participates in cytoskeletal rearrangements, cell shape changes, and motility.[21] On the other hands, some of S100A4 proteins can be secreted, and once release exert autocrine and paracrine

function modulating target cells.[22] Extracellular localization of S100A4 in rat mammary gland and secretion of this protein by rat smooth muscle cells have been reported.[20, 23] Extracellular S100A4 is a potent inducer of chemokines, metalloproteinases and urokinases.[24-26]

Rheumatoid arthritis (RA) is a most typical and severe type of chronic inflammatory disease that affects the joints and is accompanied by destruction of cartilage and bone.[27] RA is characterized by synovial hyperplasia caused by proliferating synovial lining cells and an accumulation of inflammatory cells. Synovial hyperplasia contributes to the progressive destruction of joint cartilage and bone in RA. Increased production of proteolytic enzymes, particularly matrix metalloproteinases, by activated rheumatoid arthritis synovial fibroblasts (RA-SF) is considered to be an important feature of RA pathology.[28] In contrast to non-proliferating RA-SF, proliferating RA-SF express distinct sets of genes such as S100A4.[29] Involvement of the S100 calcium binding protein family in inflammation has been suggested by several studies [16, 30]. Especially, S100A4 attract great attention since its expression is predominant in rheumatoid synovial tissues. Significantly high plasma level of S100A4 have been found in RA patients. Expression of S100A4 mRNA in the lining and sub-lining layer of RA synovium have been reported.[22] Importantly, plasma and synovial fluid of RA

patients contain S100A4 in a bioactive oligomeric conformation.[27]

The molecular role of S100A4 in the pathogenesis of RA is thought to be linked to the regulation of cell motility, adhesion, apoptosis, and proliferation, the properties that promote cancer and metastasis.[22, 31] Circulating S100A4 have been thought to modulate the modulates target cells towards a more aggressive phenotype by promoting the production of proteolytic enzymes and matrix metalloproteinases, which are required for matrix degradation and joint destruction characteristic of severe RA. Indeed, S100A4 levels directly correlate to persistent disease and to progressive bone destruction inside inflamed joints.[32] Additionally, S100A4 deficient mice are protected from subarticular bone destruction and from the systemic loss of bone mineral content during septic arthritis.[12]

Periodontitis is one of the most common oral diseases relating to the destructive inflammatory disorders of the tooth supporting tissues.[33] Periodontitis and RA have remarkably similar patho-biologic features (Table 1).[10] Several studies suggest a strong relationship between the extent and severity of periodontal disease and RA. RA patients have not only an elevated prevalence of periodontitis, but also may have severe advanced periodontitis with aggressive alveolar bone loss.[7-10] RA patient were reported to have significantly high prevalence of severe

periodontitis.[10, 34] Advanced periodontitis may also be observed in RA patients without impaired oral hygiene.[8, 10] Although taking medications that can reduce periodontal destruction such as NSAIDs and immunosuppressants, RA patients may be at risk of significant periodontal problems. Moreover, as reported in an animal arthritis model study, severe periodontal breakdown can occur without any change in oral bacteria flora.[11] However, nothing was known about the biological association between RA and periodontal disease.

Several reports have shown that S100A4 was expressed in normal periodontal tissue and localized intra- and extra-cellularly in the PDL. The study using in situ hybridization demonstrated high gene expression of S100A4 in the periodontal mesenchyme during early stages of tooth development.[35] Expression of S100A4 in PDL tissue is remarkably higher than other oral tissue such as dental follicle, dental papilla, and gingiva.[3] Moreover this expression in PDL cells is reported as high in erupted teeth whereas as faintly detectable in under-erupted teeth.[3] Furthermore, S100A4 is secreted from cultured bovine PDL cells and recombinant mouse S100A4 protein inhibit mineralized nodule formation in cultured rat osteogenic cells.[36] These results suggest physiological role for S100A4 in the PDL which relate to maintaining width of PDL space free of mineralization [3, 36]. However, little has been known about function and expression

level change of S100A4 during periodontal inflammation.

III. MATERIALS AND METHODS

3.1. Cell culture

Human PDL cells were acquired from extracted first bicuspids based on the approved protocol KHUSD 0802-01 from Kyung Hee University. The hPDL cells were cultured in α-MEM supplemented with 10% FBS and antibiotics/antimycotics at 37℃ in a 5% CO_2 incubator. Only those hPDL cells between the 4^{th} and 8^{th} passage were used in the experiments. Mouse bone marrow-derived macrophages (BMMs) were used as osteoclast progenitor cells. For BMM preparation, bone marrow cells were isolated from femurs of 5-week-old female ICR mice. After incubating overnight on culture dishes, non-adherent cells were removed. Adherent cells were further cultured for 3 days in the presence of macrophage colony stimulating factor [M-CSF (30 ng/mL)]. The cells at this stage (BMMs) were re-seeded and cultured with M-CSF (30 ng/mL) and receptor activator of nuclear factor kappaB ligand [RANKL (100 ng/mL)] to induce osteoclastic differentiation.

3.2. Cell treatment

1) Lipopolysaccharide (LPS) treatment of hPDL cells

hPDL cells were seeded to six-well plates at a density of 2×10^5 cells/well and incubated overnight to allow cells to adhere to the base of the dishes. For dose-dependency determination, the incubated hPDL cells were treated with 0, 0.1, 1.0, and 10 ng/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) and cultured for 3 or 12 h. For time-dependency experiments, the incubated hPDL cells were treated with 10 ng/mL LPS for 0-24 h. After incubation of treated cells, culture supernatant and cells were collected and analyzed by real-time RT-PCR and Western blotting.

2) Co-culture of BMMs with hPDL cells

Human PDL cells were seeded in 48-well plates at a density of 1000 cells/well. In the next day, BMMs were added to the plates at a density of 2×10^4 cells/well and cells were cultured for 1 day in the presence of M-CSF (30 ng/ml). In the following day, RANKL (100 ng/mL) was added to the wells to induce osteoclastic differentiation.

3) Recombinant S100A4 treatment of BMMs

Prior to treatment with recombinant S100A4 (ProSpec, East Brunswick, NJ,

USA), BMMs were plated in 48-well plates (2×10^4 cells/well) and cultured with M-CSF and RANKL for 1 day. Recombinant S100A4 protein (1 μg/mL) was added and cells were further cultured for 4 days. The treated cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) at day 4.

4) Signaling pathway experiments

The hPDL cells were seeded at a density of 2×10^5 cells/well in six-well plates and incubated in the absence (controls) or presence of 100 ng/mL LPS. After intervals of 0, 5, 15, or 30 min, the cell lysates were prepared and analyzed by Western blotting to determine protein levels associated with NFκB signaling (Figure 2).[37] To assess NFκB inhibition, seeded hPDL cells were pretreated with 10 μM BAY11-7085 (Santa Cruz, Dallas, TX, USA) for 2 h prior to LPS treatment. After LPS treatment, cells were collected to evaluate mRNA expression of human S100A4.

3.3. Real time RT-PCR

Total RNA was isolated by using Trizol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to complementary DNA by using SuperScript II reverse transcriptase (Invitrogen). Expression of hS100A4 was analyzed by using an

Applied Biosystems 7500 RT-PCR System with SYBR Green PCR Master Mix and gene specific primers for human S100A4: 5'-GCCCAGCTTCTTGGGGAAAA-3' and 5'-ATGGCGATGCAGGACAGGAA-3'. The protein hypoxanthine-guanine phosphoribosyl transferase (HPRT) served as the normalizer, and the relative gene expression was presented as a fold change, calculated by the ΔΔCt method. Three independent experiments were performed in triplicate for each reaction.

3.4. Western blotting

Culture supernatants or quantified cell lysates were separated by using polyacrylamide gel electrophoresis and subsequently transferred to nitrocellulose membranes. After incubating in blocking buffer (5% skim milk in Tris-buffered saline containing 0.1% Tween 20) for 1 h, the membranes were incubated with primary antibodies for overnight at 4℃. The membranes were washed and incubated with the appropriate secondary antibodies for 1 h. The immune complexes were detected by using a chemi-luminescent system. The primary antibodies used for phosphorylated and total forms of IκB, p65, pERK, p38, and JNK were from Cell Signaling Technologies (Danvers, MA, USA) and those for S100A4 and β-actin were from Abcam (Cambridge, UK) and Sigma-Aldrich,

respectively.

3.5. TRAP staining and osteoclast count

After 3 or 4 days of culture, the number of TRAP-positive cells was determined. Cells were washed in PBS, fixed in 3.7% PBS-buffered formaldehyde for 15 min, and stained for TRAP activity by using an acid phosphatase leukocyte kit (Sigma-Aldrich) according to the manufacturer's protocol.

3.6. Statistical analysis

All experiments were repeated at least three times. To determine the significance of the results, Mann-Whitney tests were performed. A p-value <0.05 was considered significant.

IV. RESULTS

4.1. LPS treatment induces S100A4 expression in hPDL cells

It was investigated whether S100A4 expression was induced in hPDL cells under conditions that mimic those in periodontal inflammation. Conventionally, those conditions can be achieved by LPS treatment of hPDL cells as reported in several *in vivo* studies.[38-40] Therefore, hPDL cells were treated with LPS varying concentration and time and expression levels of S100A4 were assessed by quantitative real-time RT-PCR analyses. As shown in Figure 3, hPDL cells showed a significant increase in S100A4 expression after both 3 and 12 h of LPS treatment. The mRNA expression of S100A4 in hPDL cells started to markedly increase at a 0.1 ng/mL LPS concentration. Compared to control sample results, 2.2-fold and 2.6-fold increases in S100A4 expression occurred at 3 and 12 h, respectively, with a 10 ng/mL LPS treatment (Figure 3A and B).

Expression of S100A4 in hPDL cells significantly increased as the duration of LPS treatment increased (Figure 4). The LPS treatment increased S100A4 expression at both mRNA and protein levels as early as after 3 h of treatment (Figure 4A and B). In addition, S100A4 secretion into the culture supernatant was

detected at 3 h after LPS treatment, and secretion was sustained at 24 h of treatment (Figure 4C). These results indicate that S100A4 expression in, and secretion from, hPDL cells are enhanced by the inflammatory stimulus of LPS treatment.

4.2. Expression of S100A4 in LPS-treated hPDL cells stimulates osteoclast differentiation

Alveolar bone resorption by osteoclasts is often associated with inflammatory periodontitis. Therefore, the effect of S100A4 up-regulation in LPS-treated hPDL cells on osteoclast differentiation was investigated. To this end, primary osteoclast progenitor cells (BMMs) were co-cultured with hPDL cells in the presence or absence of LPS (Figure 5A). In the absence of LPS, co-culture of BMM and hPDL cells resulted in a significant decrease in the number of TRAP-positive cells generated (Figure 5B). In contrast, in the presence of LPS, both monocultured BMMs and co-cultured BMM–hPDL cells showed an increase in the number of TRAP-positive cells (Figure 5C). Compared to controls, the single cultures of BMMs exhibited a 2.1-fold increase in TRAP-positive cells after LPS treatment, whereas there was an 11.5-fold increase in TRAP-positive cells in the co-cultures of BMM–hPDL cells. Notably, a large number of TRAP-positive cells were

observed around a clump of hPDL cells in co-cultures (Figure 5D).

To evaluate whether S100A4 expression in hPDL cells promoted osteoclast differentiation, BMMs were cultured in the presence of recombinant S100A4. When recombinant S100A4 was added to BMMs, the formation of TRAP-positive multinucleated cells increased (Figure 6A). In the presence of S100A4, the generation of TRAP-positive cells in BMMs was enhanced by approximately 2-fold over that in the absence of S100A4 (Figure 6B). Furthermore, S100A4 could directly stimulate the activities of ERK, p38, and JNK mitogen-activated protein kinases (MAPKs) (Figure 6C and D). These results indicate that S100A4 can directly promote the differentiation of BMMs into osteoclasts, at least in part by stimulating MAPKs.

4.3. Involvement of NFκB pathway in LPS-induced S100A4 expression in hPDL cells

The NFκB signaling pathway has been shown to mediate LPS induction of protein expression in many genes.[41] Therefore, it was investigated whether NFκB pathway was involved in the LPS induction of S100A4 expression in hPDL cells. To assess LPS activation of the NFκB signaling pathway in hPDL cells, the

phosphorylation of IκB and p65 proteins were monitored. The phosphorylated form of IκB increased a few minutes after LPS treatment and reached the highest level 30 min after LPS treatment. Accordingly, the total protein level of IκB slightly decreased after LPS treatment (Figure 7A). The phosphorylated form of p65 in hPDL cells also increased in a manner similar to that of IκB after LPS treatment while the total p65 level in hPDL cells was not affected by LPS treatment (Figure 7A).

To investigate whether the NFκB signaling pathway mediated S100A4 production, hPDL cells were treated with LPS in the presence of BAY11-7085, an NFκB inhibitor. When the hPDL cells were treated with BAY11-7085, LPS-induced S100A4 expression was negated (Figure 7B).

V. DISCUSSION

Cells of the PDL, which lies between alveolar bone and cementum, are proximal to oral bacterial flora. A major component of the outer membrane of Gram-negative bacteria, LPS, also known as lipoglycans or endotoxin, is reported to stimulate inflammation.[42] Therefore, in this study LPS was used to create inflammatory conditions in hPDL cells. In periodontitis, expression of specific genes and production of various proteins within PDL cells are altered.[41, 43] In our study, S100A4 expression was significantly increased in hPDL cells under periodontal inflammation conditions that were mimicked by treatment with LPS (Figures 3 and 4). LPS treatment of hPDL cells led to an increase in S100A4 expression in both dose- and time-dependent manners.

Most S100 proteins are synthesized and localized intracellularly. While many S100 proteins exert regulatory functions within cells, some are secreted and play cytokine-like roles by modulating target cells.[22] Immunolocalization determined by using cryosections and analysis of PDL cell cultures has revealed that PDL cells secrete S100A4 protein both *in vivo* and *in vitro*.[36] In the present study, elevated S100A4 protein levels were observed in both cell lysate and culture supernatant. These results indicate that periodontal inflammation conditions could

not only result in an increased intracellular S100A4 level in hPDL cells, but could also elevate S100A4 secretion from hPDL cells.

RA and periodontitis are chronic inflammatory disorders characterized by deterioration of bone structures and in which pro-inflammatory cytokines stimulate osteoclast differentiation and activity. In the case of RA, an increase in S100A4 expression has been shown to be associated with progressive bone destruction.[32, 44, 45] In S100A4 knock-out mice, the number of functional osteoclasts was reduced and there was a substantial increase in bone mass.[21] These reports suggest that an increase in S100A4 expression, which occurs in RA and other inflammatory diseases, may in part be responsible for high bone turnover and resorption rates. In the present study, co-culture experiments were performed to investigate the effect of S100A4 in LPS-treated hPDL cells on osteoclastogenesis (Figure 5). In the absence of LPS, BMMs co-cultured with hPDL cells resulted in a significant reduction in the formation of TRAP-positive osteoclasts. That result is probably due to competition between the two cell types. In the presence of LPS, monocultured BMMs showed enhanced osteoclast differentiation, which supports the results in other studies.[46, 47] LPS-dependent increase in osteoclastogenesis also occurred in the BMM-hPDL co-culture indicating that the suppressive effect of the hPDL cells was overridden. Moreover,

the extent of the LPS effect was much higher in the co-culture samples. Based on these results, it was investigated whether exogenous S100A4 could affect osteoclast differentiation. Direct treatment of recombinant S100A4 increased the formation of TRAP-positive osteoclasts from BMMs (Figure 6). Therefore, the increase in S100A4 expression in hPDL cells appears to be a possible pathogenic linkage between RA and advanced periodontitis.

Extracellular S100A4 induces cell responses by binding to surface receptors. The receptor for advanced glycation end products (RAGE) has been shown to mediate S100A4 function in various cell types including monocytes/macrophages.[48] In addition, S100A4 increased MMP13 production from human articular chondrocytes via RAGE.[26] This response was associated with the phosphorylation of Pyk-2 and MAPKs and the activation of NF-κB.[26] In this study, the stimulation of MAPK signaling pathways by S100A4 was detected in differentiating osteoclasts. Whether other signaling mechanisms and the RAGE receptor are involved in the stimulatory effect of S100A4 on osteoclastogenesis is one of the subjects of our next studies.

LPS has been reported to induce expression of pro-inflammatory cytokines via the NFκB pathway.[41, 49] The NFκB family is comprised of dimeric complexes of

five NFκB proteins (p50, p65, c-Rel, p52, and RelB), which are combined with the inhibitory protein IκB during the inactive state. Activation of NFκB, which is induced by a variety of extracellular signals, can be regulated by phosphorylation of IκB or p65 proteins. Phosphorylation of the IκB protein leads to ubiquitination and self-degradation, which results in translocation of NFκB to the nucleus. In the nucleus, NFκB binds to a specific sequence and positively regulates the transcription of genes related to pro-inflammatory cytokines. The phosphorylation of p65 increases the transcriptional activity of NFκB in the nucleus.[50-52] In the present study, LPS treatment of hPDL cells resulted in increased phosphorylation and degradation of IκB. In addition, LPS treatment of hPDL cells increased the level of phosphorylated p65. Thus, LPS treatment of hPDL cells can induce activation of the NFκB pathway. In addition, BAY11-7085, an inhibitor of the NFκB pathway, almost completely blocked the induction of S100A4 expression by LPS in hPDL cells (Figure 7). Therefore, the NFκB signaling pathway is the critical mechanism that mediates S100A4 up-regulation by LPS in hPDL cells.

Patients who are suffering from systemic disease are at greater risk for complications during orthodontic treatment. RA is one of the most problematic disease that may complicate orthodontic treatment. Juvenile rheumatoid arthritis frequently produces severe mandibular deficiency, and adult onset rheumatoid

arthritis can destroy the condylar process and create a deformity.[53] These skeletal problems accompanied with RA are so severe that major considerations have been focused on the skeletal aspects, in fact.[54, 55] Periodontal problem is another aspects not to be overlooked when orthodontist treat RA patients. RA patients have not only an elevated prevalence of periodontitis, but also may have severe advanced periodontitis with aggressive alveolar bone loss.[7-10] Moreover, destructive periodontal breakdown can occur without impaired oral hygiene.[8, 10, 11] It is assumed that aggressive pattern of periodontitis in RA patient is related to underlying dysfunction of inflammatory mechanisms which is common in two diseases.[34] From our results, S100A4 appears to be a possible factor associated with such dysfunction. Increased osteoclastic activity induced by elevated S100A4 may intensify periodontal problems during orthodontics. Prolonged orthodontic treatment should be avoided in RA patients because the potential for harm is at least as great as the potential benefit.

VI. CONCLUSIONS

Inflammation associated with periodontitis increases S100A4 expression in hPDL cells via activation of the NFκB signaling pathway. Extracellular S100A4 secreted from hPDL cells after stimulation by inflammatory conditions may enhance osteoclast differentiation, which can contribute to bone destruction.

Table 1 Possible interrelationships between rheumatoid arthritis and periodontal disease (modified from Mercado, 2000)[10]

Pathogenesis of Rheumatoid Arthritis	Pathogenesis of Periodontal disease
Chronic inflammatory disease	
Immunoregulatory imbalance	
Role of bacteria/peptide as adjuvant antigen in auto-antibody production	Periodontal pathogens as etiological agent
HLA-DR antigen association	
Persistence of antigen/peptide in progressive type	Persistence of periodontal pathogens in progression
Role of macrophage and dendritic cells	
Interleukin-1, tumor necrosis factor-α, prostaglandin-E2	
Th1=Th2	Th1, ↑ Th2
Role of Nitric Oxide in pathogenesis	
Genetic and environmental influences	

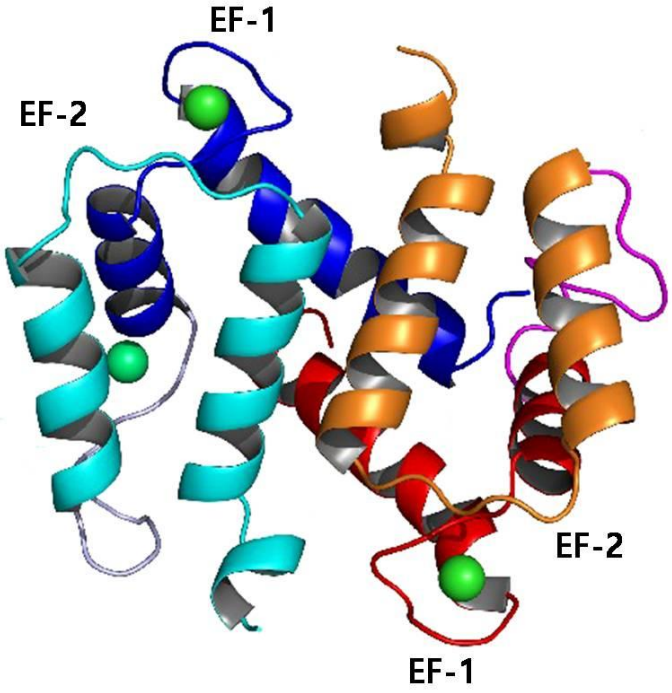

Figure 1 Dimer structure of S100A4 proteins. S100A4 protein has two EF-hand type calcium-binding motifs; one canonical and another specific to the S100 family. (modified from Heizmann, 2002)[14]

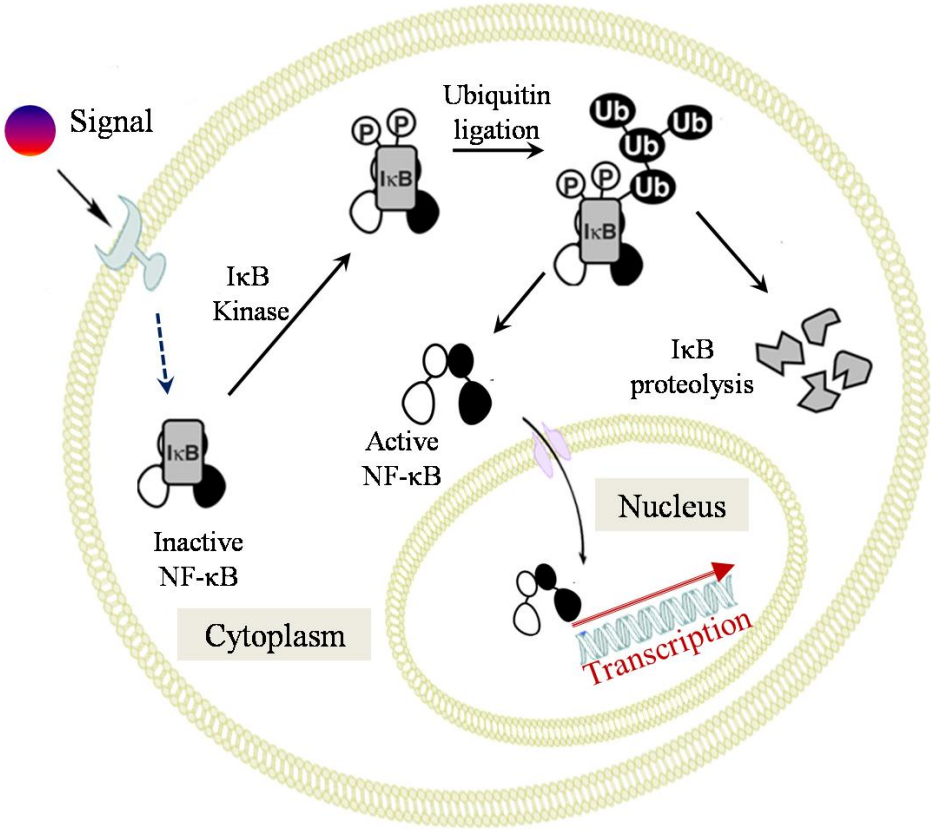

Figure 2 NF-κB signaling. Activation of NFκB, which is induced by a variety of extracellular signals, can be regulated by phosphorylation of IκB or p65 proteins. Phosphorylation of the IκB protein leads to ubiquitination and self-degradation, which results in translocation of NFκB to the nucleus. In the nucleus, NFκB binds to a specific sequence and positively regulates the transcription of genes related to pro-inflammatory cytokines. (modified from Gilmore, 2006)[37]

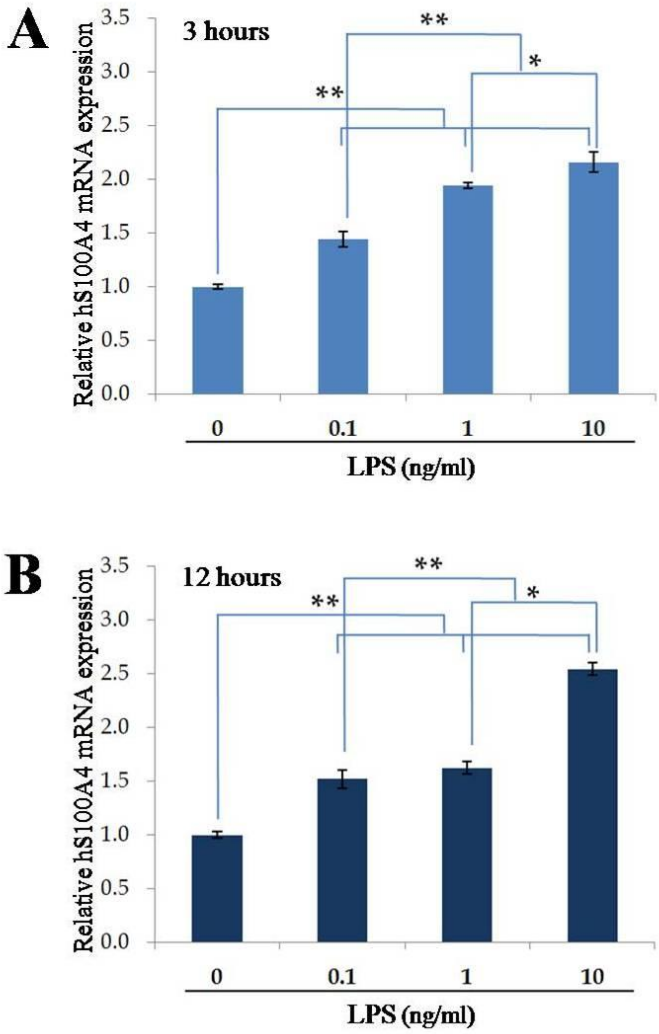

Figure 3 The mRNA expression of S100A4 in hPDL cells was increased in a dose-dependent manner following treatment with LPS. (A) The hPDL cells treated with the different concerntrations of LPS for 3 hours induced an increase of S100A4 expression. (B) The hPDL cells treated with the different concerntrations of LPS for 12 hours induced an increase of S100A4 expression. ($^{*}P< 0.05$, $^{**}P< 0.01$)

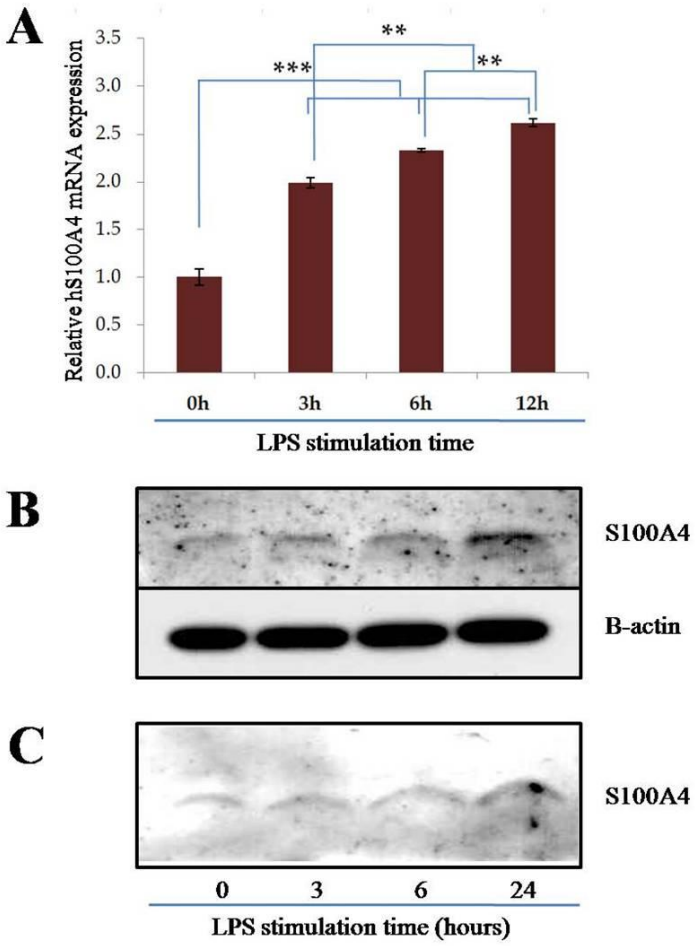

Figure 4 The expression of S100A4 in hPDL cells was increased in a time-dependent manner following treatment with 10 ng/ml of LPS. (A) The expression of S100A4 in hPDL cells was increased as the duration of LPS treatment increased. (B) The protein level of S100A4 in the whole cell lysate was increased as the duration of LPS treatment increased. (C) The protein level of S100A4 in culture spernatants was increased as the duration of LPS treatment increased. ($^{**}P< 0.01$, $^{***}P< 0.001$)

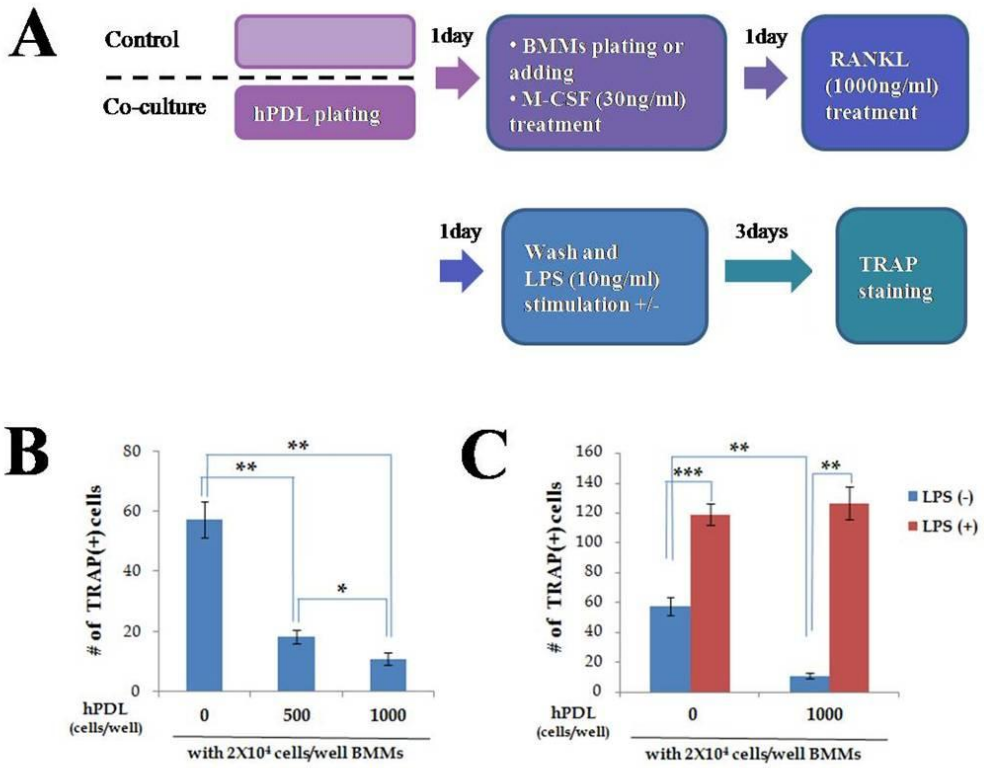

Figure 5 LPS-induced S100A4 expression in hPDL cells stimuated osteoclast differentiation. (A) BMMs co-cultured with hPDL cells was performed as followings. (B) In the absence of LPS treatment, the number of TRAP-positive cells was decreased with increasing density of hPDL cells. (C) In the presence of LPS treatment, the number of TRAP-positive cells was increased in BMMs co-cultured with hPDLs. (D) A large number of TRAP-positive cells were observed around LPS treated hDPL cells. ($^{*}P< 0.05$, $^{**}P< 0.01$, $^{***}P< 0.001$)

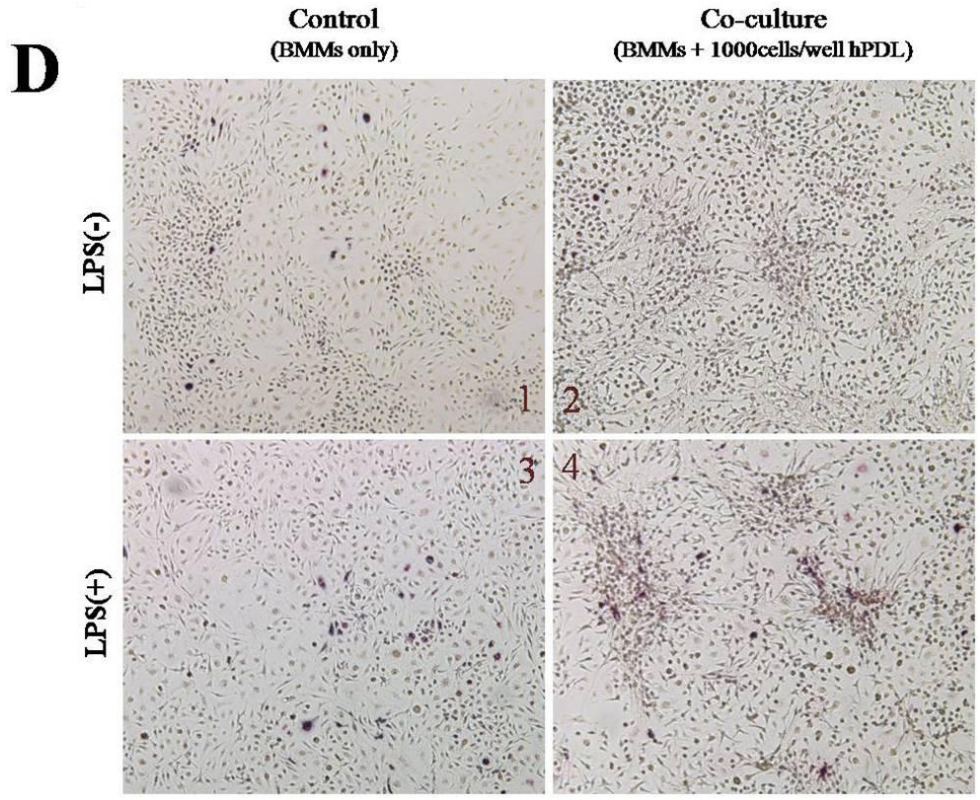

Figure 5 Continued

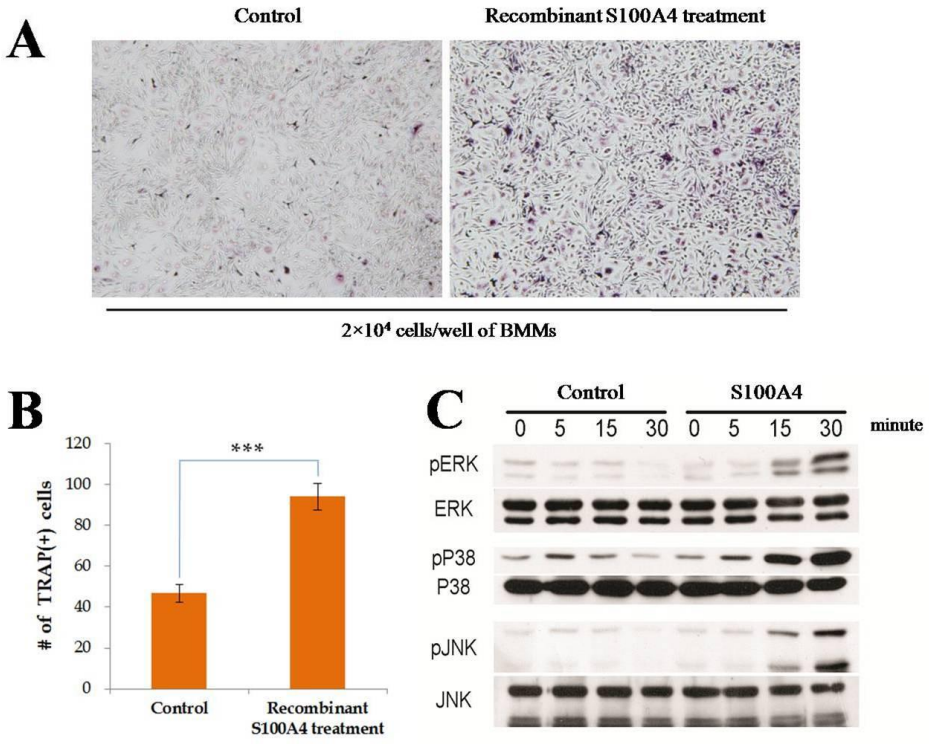

Figure 6 Recombinant S100A4 increased osteoclast generation from BMMs. (A) The formation of multinucleated cells was increased in BMMs treated with recombinant S100A4. (B) The number of TRAP-positive cells generated was higher in BMMs treated with recombinant S100A4 compared to vehicle-treated BMMs. ($^{***}P< 0.001$) (C) The activities of ERK, p38, and JNK measured by Western blotting for the phosphorylated form of each MAPK were stimulated by S100A4. (D) Binding of RANKL to its receptor RANK leads to recruitment of the adaptor molecules TRAF6 and activation of MAPK pathway. These signaling result in increased bone resorption via promoting osteoclast differentiation. (modified from Lewiecki, 2011 and Wang, 2014)[56, 57]

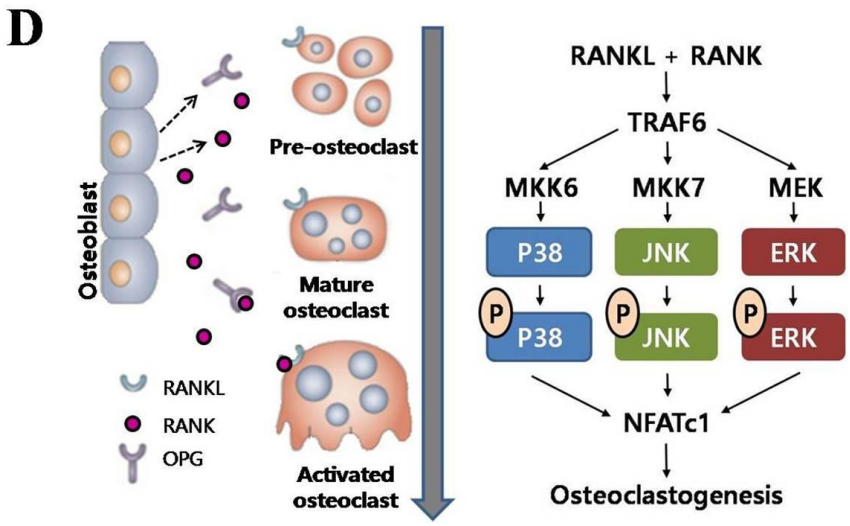

Figure 6 Continued

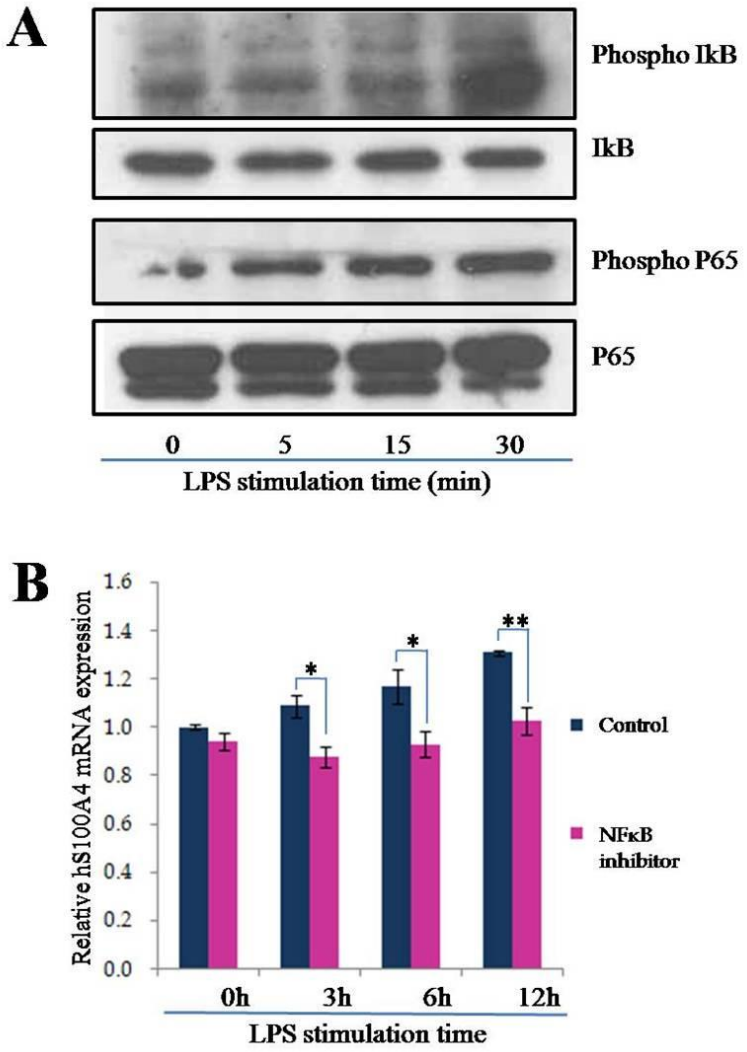

Figure 7 NFκB pathway was involved in LPS-induced S100A4 up-regulation in hPDL cells. (A) The phosphorylated form of IκB was increased in hPDL cells after treatment with LPS. The phosphorylated form of p65 in hPDL cells was increased following LPS treatment. (B) S100A4 induction in hPDL cells by LPS was blocked by treatment with the NFκB inhibitor, BAY11-7085. ($^{*}P< 0.05$, $^{**}P< 0.01$)

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국문초록

치주인대세포의 S100A4 발현 증가가 파골세포 형성에 미치는 영향

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목 적:

다양한 염증성 질환에서 S100A4의 발현이 증가한다는 것은 여러 연구에서 보고된 바 있다. 하지만 치주질환과 S100A4의 관련성에 대해서는 알려진 바가 적다. 본 연구에서는 염증성 자극이 가해졌을 때 인간 치주인대세포 내에서의 S100A4 발현이 어떻게 변화하는지 살펴보고, 가능한 메커니즘을 도출하고자 한다.

방 법:

인간 치주인대세포에 지질다당류 (lipopolysaccharide; LPS)를 처리하여 염증성 자극을 가한 후, S100A4 발현의 변화를 real-time RT-PCR 과 Western blotting 을 이용하여 분석하였다. 파골전구세포와

인간 치주인대세포를 공동 배양하는 환경에서 LPS 를 처리하였을 때, 파골세포 분화 정도를 평가하였다. 또한 파골전구세포에 직접적으로 재조합 S100A4 를 처리한 후 파골세포 분화 정도를 관찰하였다. Western blotting 을 통해 인산화 형태의 IκB (inhibitor kappa B)와 p65 를 검출하여 NFκB (nuclear factor kappa B)의 활성도를 평가하였다. LPS 를 처리한 인간 치주인대세포에 NFκB 억제제를 첨가하였을 때 S100A4 의 발현 변화를 RT-PCR 을 통해 측정하였다.

결 과:

LPS를 처리할 경우 인간 치주인대세포에서 S100A4의 발현이 유의하게 증가하였을 뿐만 아니라, 세포에서 S100A4 단백질 분비도 증가하였다. 염증성 환경에서 인간 치주인대세포의 S100A4 발현 증가는 파골세포 형성을 촉진하였다. 또한 재조합 S100A4를 직접적으로 처리한 경우에도 파골세포 분화를 촉진하였다. 인간 치주인대세포에서 S100A4 발현의 증가는 NFκB 신호 기전의 활성화와 관련된다.

결 론:

본 연구 결과는 치주 질환에서 나타나는 골 파괴 양상이, 인간 치주인대세포 내의 S100A4 발현 증가와 관련될 수 있음을 시사한다.

주요어: S100A4, 치주인대세포, 파골세포 형성, 염증, NFκB

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